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Genetics

Mitochondrial DNA heteroplasmy in *Drosophila mauritiana*

(extrachromosomal inheritance/physical mapping/sequence repeats)

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ABSTRACT **Mitochondrial DNA extracted from an isofemale strain of *Drosophila mauritiana* (subgroup *melanogaster*) appeared to be heterogeneous in size. A short genome [S; 18,500 base pairs (bp)] and a longer one (L; 19,000 bp) coexist in the preparation. The additional 500 bp have been located within the A+T-rich region. *Hpa* I digest patterns suggest that the S genome may carry a duplication of a 500-bp sequence including an *Hpa* I site and that the L genome may carry a triplication of the same sequence. At the 30th generation of the isofemale strain, 60 female genotypes were examined individually. Half of the flies were pure either for the S or the L DNA. The remaining 50% exhibited various degrees of heteroplasmy for the two DNA types. Among metazoan animals, this *D. mauritiana* strain offers an exceptional situation with regard to the number of individuals heterogeneous for mtDNA and the relative stability of heteroplasmy through generations.**

In metazoan animals, the lack of mitochondrial mutants is compensated by the use of restriction endonucleases to study the heredity and the evolution of mitochondrial DNA. Three general features of animal mitochondrial genetics emerge from different analyses (1). (*i*) mtDNA is maternally inherited: the first evidences from interspecific hybridization experiments (2, 3) have been confirmed later by intraspecific crosses. (*ii*) Sequence differences are frequent between individuals within the same species and even within the same population (4–8). (*iii*) From the existence of interindividual differences, a heteroplasmic transitory phase might be expected; however, for each individual the mtDNA always appears as a molecular clone (6–10): the mtDNA is homogeneous for a given organ, and the DNA molecules are identical from one organ to another within an individual (4, 11). This last observation suggests a rapid purification of the mitochondrial genome.

Drosophila mtDNA exhibits these general properties (12–14). In the course of a sequence variability study within the *melanogaster* subgroup of *Drosophila*, we analyzed numerous isofemale strains. As expected, the mtDNA from most of them was homogeneous. But one *D. mauritiana* strain studied, although initiated from a single fly, provided a heterogeneous mtDNA: two types of molecules different in length have been detected. We determined individual mitochondrial genotypes in terms of DNA length of 60 flies. Some individuals only possess one DNA type, whereas others, carrying both types in different proportions, are heteroplasmic. In order to characterize and compare the two genomes, the physical map of both DNAs was established: the length difference corresponds to various degrees of sequence repetition in the A+T-rich region.

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MATERIAL AND METHODS

D. mauritiana, endemic in Mauritius Island (15), is one of the eight species of the *melanogaster* subgroup (*Sophophora* subgenus). The original strain (163-1) was established in July 1973 with individuals collected at Chaland. Our strain (H1) was initiated from one single fly and has been maintained in mass culture for 30 generations.

For physical mapping, mitochondria were isolated either from embryos, from virgin eggs, or from adult flies. mtDNA was purified on CsCl gradients. If necessary, a second purification was carried out on CsCl/4′,6-diamidino-2-phenylindol-2·HCl (DAPI) gradients. Cleavage sites of nine restriction endonucleases were mapped by analysis of partial and complete single digests and of double digests. The digestion fragments were separated by vertical electrophoresis on 1% agarose or 4.5% acrylamide slab gels. *Hin*dIII/λ phage and *Hin*dII/φX174 phage DNA digests were used as molecular weight standards for calibration. After electrophoresis the gels were stained by standard procedures with ethidium bromide and then photographed under short wave UV light.

For individual mitochondrial genotype comparison, it was not possible to get from a single fly enough DNA for a slab gel analysis; so we used the progeny of each female fly under study. About 30 generations after our strain initiation, 60 impregnated female flies were isolated individually in culture vials; then F_1 flies were transferred into bottles. The F_2 pupae were taken out, and only the females were allowed to emerge. Total nucleic acids were extracted from virgin eggs laid by these females. Because in unfertilized eggs a great part of the DNA comes from mitochondria, no further purification was required except occasional RNase treatment; a few micrograms of mtDNA usually were obtained from about 10,000 eggs. Each "individual" DNA sample was digested by *Hae* III and treated as described above. Negative films of the gel pictures were then analyzed by densitometry.

RESULTS

mtDNA Heterogeneity at the Population Level. mtDNA from the H1 isofemale strain of *D. mauritiana* (established for 30 generations) was digested by nine multiple-site enzymes (Fig. 1). For each digest the fluorescence of most bands, measured as the surface of the peaks on a densitometer profile, was proportional to the size of the corresponding fragment, and a one-to-one molecular stoichiometry was found among them. But two close bands were systematically underrepresented (except with *Hpa* I, see below); however, their sum fits with the general stoichiometry (Fig. 2). This reflects the existence of two molecular forms in the preparation.

Furthermore, the migration difference of the two nonstoi-

Abbreviation: bp, base pairs.

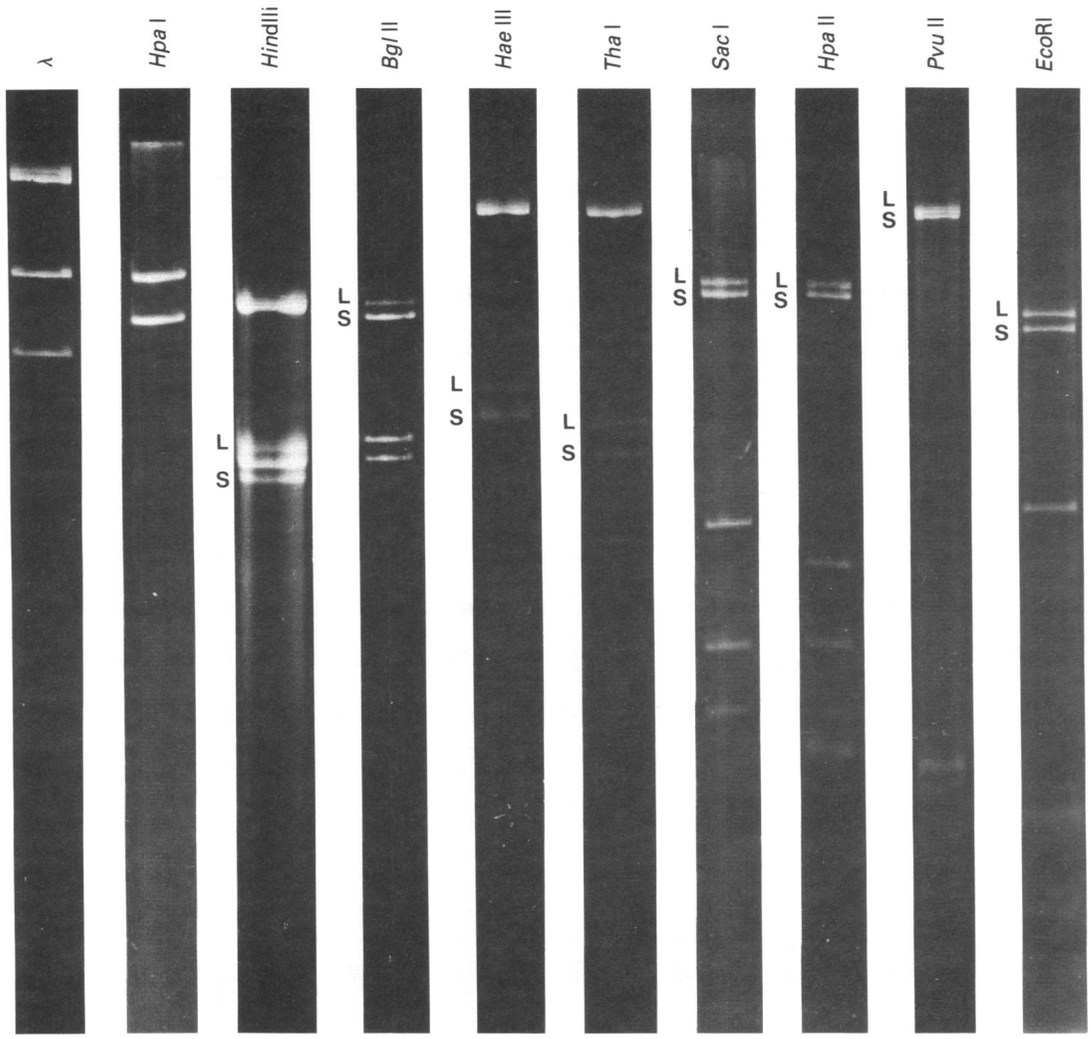

FIG. 1. Digest patterns of *D. mauritiana* mtDNA with nine restriction enzymes. The mtDNA, extracted from a population, is heterogeneous in length. Each enzyme (except *Hpa* I) exhibits two nonstoichiometric bands (L and S) generated by the long and the short genome, respectively. *Hin*dIII/λ phage DNA digest was used as a molecular weight standard.

chiometric bands corresponds to a constant length difference of 500 base pairs (bp) from one enzymatic profile to another. Thus, the preparation contains two types of molecules: a short genome (called S) measuring 18,500 bp and a long one (named L) measuring 19,000 bp. Because this observation was performed at the population level, it was tempting to investigate mtDNA heterogeneity in individual flies.

mtDNA Heterogeneity at the Individual Level. To reveal individual genotypes, two procedures could be followed—either a direct study of mtDNA from one fly (but the small quantity of DNA would require micromethods with labeled DNA) or, taking advantage of the maternal inheritance of the mitochondrial genome, the analysis of mtDNA pool from single female progeny. This latter procedure was used.

Each "individual" DNA sample was digested by *Hae* III; this enzyme recognized only two cleavage sites on the mtDNA molecules. It generated a large fragment (Fig. 3, fragment A; 12,800 bp) and a smaller one (fragment B), the size of which was variable: *Hae* III B_S = 5,630 bp and *Hae* III B_L = 6,130 bp, easily distinguished on gels. This *Hae* III digestion was used routinely to characterize the occurrence of the S and L types in a mtDNA preparation.

Of the 60 mtDNAs analyzed, 33 profiles are shown in Fig. 3. Some of them (18D, 20C, 26B, . . .) exhibited both the S and L mtDNA types; others appeared to be homogeneous either for the S type (29C, 18C, 25D, . . .) or for the L one (19A, 21D, 22B, . . .).

As described earlier, for every isofemale line, mtDNA was prepared from pooled F_3 eggs. Because a DNA heterogeneity was detected, a heteroplasmic state of the initial female could be inferred. To support this assumption, one has to show that the estimate of the L and S mtDNA percentage in the F_3 egg preparation is the same as in the ancestral female cells. In order to clarify this important point, the progeny of a single putative heteroplasmic female (18D, 48.5% L DNA) was analyzed: F_3 females were isolated and their genotype was established by the above procedure. For each of the 31 descendants analyzed, the mtDNA was clearly heterogeneous. The average of the L DNA percentage distribution was 47.6%; this value is close to the one established at the F_3 egg level (48.5%). Consequently, a mitochondrial female genotype can be quantitatively appraised through the DNA extracted from pooled eggs of later generations. Thus, this procedure allows us to reveal the existence of heteroplasmy, to quantify it, and to follow its persistence and evolution through generations.

In the original isofemale strain (H1), 30 of the 60 flies ana-

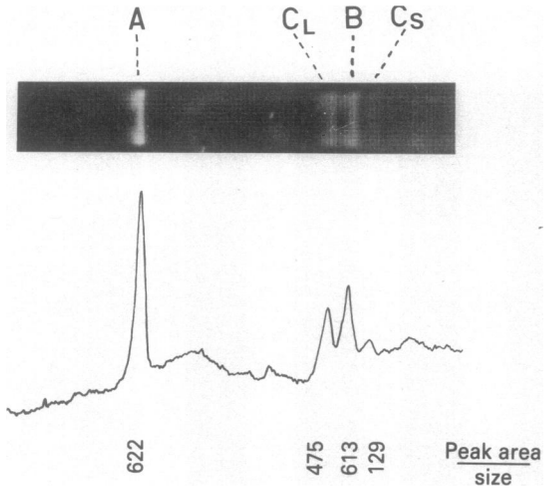

FIG. 2. Densitometric profile of the *Hin*dIII digest. The C_S and C_L fragments (4,580 and 5,080 bp, respectively) appear to be nonstoichiometric to A and B from a comparison of the peak area/size ratio. The sum of the ratios for C_S and C_L equals that of either A or B.

lyzed were heteroplasmic with various L DNA proportions (2% being the technical detection limit); 14 and 16 individuals were considered as homoplasmic, respectively, for the S and L DNA.

Localization of the Variable Region on the Physical Map. Using two enzymes (*Eco*RI and *Hin*dIII), Fauron and Wolstenholme (13) have constructed a physical map of *D. mauritiana* mtDNA. We extended this map with seven additional enzymes. In fact, two maps have been established, depending on the genome size. For each enzyme (except *Hpa* I), all the cleavage sites are strictly identical on the two maps. Only the shorter genome map is drawn in detail (Fig. 4). The length difference (500 bp, see above) always affected a fragment mapped in the part of the genome that includes the A+T-rich region previously located by electron microscopy (13). The location of this difference between S and L types can be mapped precisely by use of *Hpa* I: in the A+T-rich region, the S DNA possesses two *Hpa* I sites delimiting a 500-bp fragment; the L DNA has an additional *Hpa* I site, generating another 500-bp fragment adjacent to the previous one.

DISCUSSION

Size Variation of mtDNA. The mitochondrial genomes of metazoan animals are relatively homogeneous in size, whereas their lengths vary widely among eukaryotes (16, 17). Among mammalian species, small differences have been located in the D-loop region (5, 18–20). In *Drosophila* large interspecific variations may affect the A+T-rich region (12, 21–23), which overlaps the origin of replication (24, 25). Recently, length variations also have been detected within three *Drosophila* species (13, 14): *D. melanogaster*, *D. simulans*, and *D. mauritiana*. In this last species, Fauron and Wolstenholme (13) have observed two mtDNA types (I and II) that differ in size (700 bp) and *Eco*RI digests (type I, eight sites; type II, seven sites). The present study detected two mtDNA types, called S and L, which are themselves different in size (500 bp) but identical for the *Eco*RI pattern (eight cleavage sites). According to the number, the

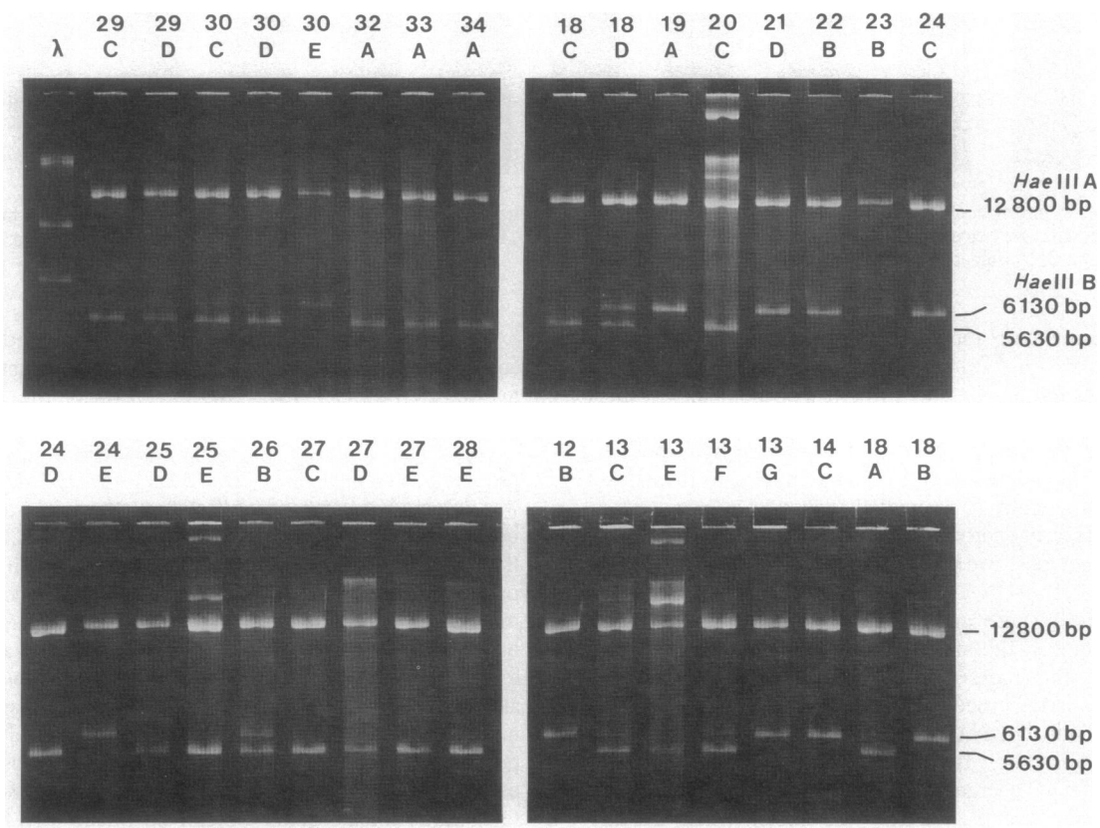

FIG. 3. mtDNA heterogeneity at the individual levels. *Hae* III digest products of individual mtDNA (extracted from F_3 eggs; see text for discussion) were separated on 1% agarose gels. The genome possesses two sites for this enzyme; the heterogeneity affects the B fragment. Some individuals are pure either for a slow-mobility fragment (6,130 bp; 19A, 21D, 22B, . . .) or for a fast one (5,630 bp; 29C, 18C, 25D, . . .). Others are heteroplasmic and exhibit the two forms of the B fragment in different proportions (18D, 20C, 26B, . . .). Because of a DNA excess, the samples 20C, 25E, and 13E are not digested to completion.

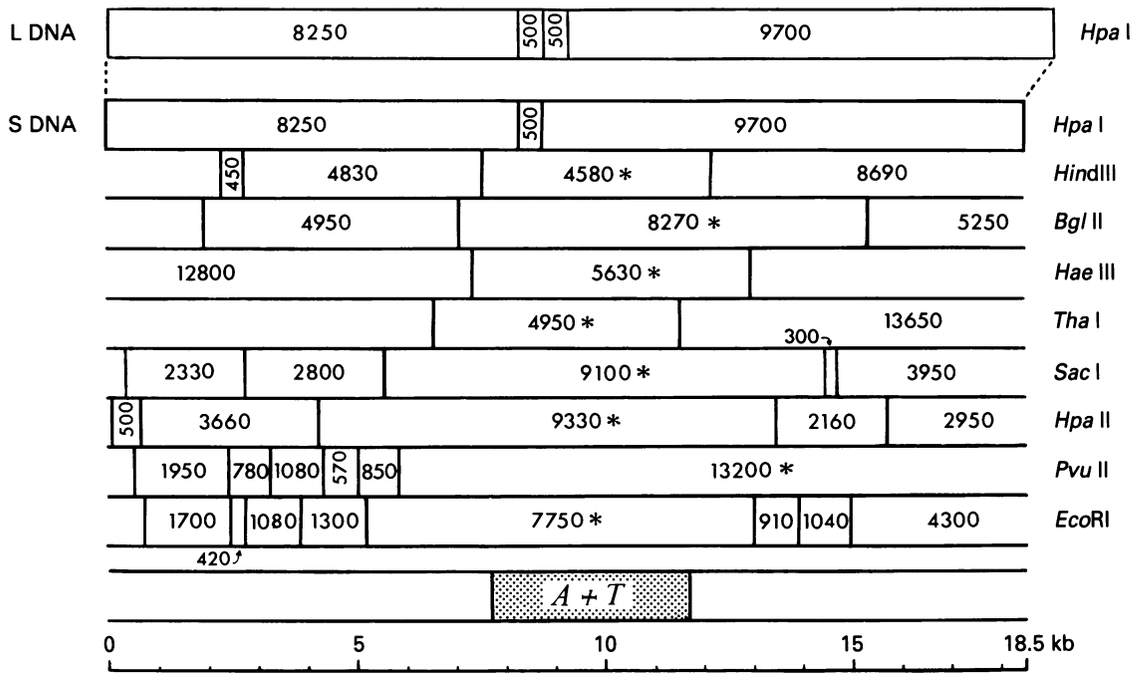

FIG. 4. Physical map of *D. mauritiana* mitochondrial genome. The circular genome has been linearized on the *Hpa* I-sensitive site present outside of the A+T-rich region. A detailed map of the short genome (S DNA) is shown: 38 cleavage sites, generated by nine enzymes, were mapped. Except for *Hpa* I, the L DNA exhibits the same restriction sites as in the S DNA, but all fragments, including the A+T-rich region (*), are 500 bp longer: the L DNA possesses an additional 500-bp *Hpa* I fragment (upper line), which accounts for the length difference between S and L DNA. The A+T-rich region has been drawn according to Fauron and Wolstenholme (13).

length, and the mapping of the *Eco*RI fragments, our L type might be identical to the type I described earlier (13). However, a comigration of the two DNAs would be necessary to ensure their homology, especially in the A+T-rich region.

The physical mapping of *D. mauritiana* mtDNA revealed the existence of several *Hpa* I sites within the A+T-rich region. Because of its base composition, the probability that any restriction site lies in this region is very low. In fact, among all the previously mapped sites in various *Drosophila* mtDNA (12, 13, 26, 27, 28), only three (*Eco*RI and *Hpa* I in *D. yakuba* and *Hin*fI in *D. virilis*) have been localized in this part of the genome. The presence of two *Hpa* I sites (type S) and even three sites (type L) within the A+T-rich region of the *D. mauritiana* genome can be explained by a repeat unit of 500 bp, including one *Hpa* I site. The S DNA could carry a duplication of this unit, and this would account for the 500-bp fragment in *Hpa* I digests. A triplication of the same sequence, probably organized in direct tandem repeat, would generate two identical 500-bp fragments observed in the *Hpa* I digest of the L DNA. Sequence repeats in the A+T-rich region already have been suggested (21, 27, 29), although the structure of this part of the genome is not yet understood completely.

Individual Heterogeneity of mtDNA. In some organisms (yeast, *Paramecium*, *Oenothera*, *Pelargonium*) with biparental cytoplasmic heredity, organelle heteroplasmy occurs whenever the two parents are genetically different. However, after a few generations, individuals become homogeneous again: the heteroplasmy, easily obtained, is never maintained for a long time (30).

In many species mitochondrial heredity is strictly maternal and heteroplasmy can only be created by mutations affecting the female germ line. After the occurrence of a mutation, a heteroplasmic state must exist. Very often, accurate analysis has failed to detect any intraindividual heterogeneity (6, 8, 11).

Only a few examples of such a state have been reported (4, 31–33). The occurrence of a mtDNA polymorphism in a maternal lineage of Holstein cows has been an advantageous situation for the analysis of heteroplasmy; however, it has not been observed *per se* in any animal (34). In the present study, the analysis of 60 *D. mauritiana* females revealed an intraindividual heterogeneity of mtDNA for half of the flies.

A length variation is easier to detect than a base substitution: a length difference is apparent on any restriction profile, whereas the probability to detect a single base change through restriction enzyme analysis is low. This consideration does not hold for the Holstein cows study where an *Hae* III site difference has been identified (34).

The discrepancy between the observations in the study of Holstein cows and in *Drosophila* could be related to the nature of the cells used to prepare mtDNA—somatic cells instead of eggs. If it is so, some segregation system would operate in oogenesis or early embryogenesis.

Other explanations for our observation can be searched for in the fact that heteroplasmy depends on mutation frequency and length of the period required for fixation. As it is rarely detected, the heteroplasmic state has been thought to be fugacious. In *D. mauritiana* it seems to be maintained for a relatively long period. Its persistence for at least three generations has been demonstrated through the 18D line analysis. The initial female fly (H1) was probably heteroplasmic itself because the mtDNA has been detected as heterogeneous a few generations after foundation of the strain, and furthermore, the heteroplasmy has persisted over 30 generations (at least). However, *D. mauritiana* may not be exceptional in spite of its heteroplasmic state length; the scarcity of heteroplasmy observations in other organisms may correspond to longer delays between mutations. In this case, *D. mauritiana* would only be singular for a high mutation rate.

From now on this *D. mauritiana* strain appears to be a suitable material for further experiments on organelle heredity in

metazoan animals. It remains unknown whether the present situation is peculiar to this species or is relevant to general rules of extrachromosomal inheritance.

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